Induction of ovulation in rabbits by adding Lecirelin to the seminal dose: *In vitro* and *in vivo* effects of different excipients

A. Dal Bosco , R. Cardinali , G. Brecchia , P.G. Rebollar , M. Fatnassi , P. Millán , S. Mattioli , C. Castellini

Keywords:
Rabbit
Lecirelin
Intravaginal administration
Ovulation
Fertility

A B S T R A C T

This study evaluates the effect of Lecirelin (Dalmarelin®, Fatro, Italy) diluted in different excipients (benzilic alcohol, benzoic acid and paraben) added to a seminal dose on LH concentrations, progesterone concentrations and ovarian status in rabbits. The *in vitro* effect on spermatozoa was also tested. A total of 100 multiparous female rabbits were divided into 5 groups, which at the moment of AI, received 0.2 mL (5 μg/dose) intramuscular (im) inoculation of Lecirelin (control) or the same Lecirelin dose administered intravaginally (iv) with the seminal dose alone (Lecirelin group) or with benzilic alcohol (Lecirelin BA group), benzoic acid (Lecirelin BAc group) or parabens (Lecirelin PA group) as an excipient. After 7 days, 10 rabbits per group were euthanized to analyze their ovarian status. In the control group, a high LH peak was detected 30 min post AI, while in the iv groups a slight increase in LH occurred after 120 min. The ovulation and fertility rate was similar in control and Lecirelin groups, while the lowest fertility rate was detected in the Lecirelin BA group. In a second experiment, the semen samples collected from male rabbits were diluted in TALP (control) or mixed with the 5 μg of Lecirelin solutions used in the first experiment. The highest percentage of capacitated sperm (68.3%) was recorded in the Lecirelin PA. The lowest percentages were observed in the Lecirelin BA and BAc groups. In conclusion, the iv administration of Lecirelin represents an alternative method for simplifying rabbit insemination procedures.

1. Introduction

Artificial insemination (AI) is employed for intensive rabbit breeding (Castellini et al., 2010). This technique is widely used in many European countries, such as Italy, France, Spain and Germany (Theau-Clément, 1998). AI offers improved genetic selection, prolonged fertility and simplified management of the cycled production system (Theau-Clément et al., 2006).

The rabbit is a reflexively ovulating species. Rabbit ovulation is induced by natural mating and/or by a synergy between sensory and neuroendocrine stimuli (Rebollar et al., 2012).

The most frequent method used for ovulation induction in artificially inseminated rabbits is the intramuscular (im) administration of GnRH analogs (Theau-Clément et al., 1990; Rebollar et al., 1997). This method can be stressful for the animal and requires a significant amount of work and time on the part of the operators (Dal Bosco et al., 2011; Vicente et al., 2011). Recent studies have investigated the possibility of ovulation induction in rabbits by mucosal absorption after supplementation of a semen extender with GnRH synthetic analogs (Quintela et al., 2004, 2009; Viudes-de-Castro et al., 2007; Ondruska et al., 2008; Vicente et al., 2008), which simplifies the insemination procedure.

Quintela et al. (2004) showed that the addition of intravaginal Buserelin to the seminal dose (16 μg/rabbit) provides a similar result to im treatment. The inoculation of GnRH-Lecirelinum (7.5 μg/rabbit) with the seminal dose, induced ovulation and improved kindling rate compared to the control (im) (Ondruska et al., 2008). In a control study, Viudes-de-Castro et al. (2007) showed that the addition of different GnRH analogs to seminal doses achieve an ovulation response similar to im treated animals. Nonetheless, Vicente et al. (2008) reported that the intravaginal supplementation of semen with 10 μg/mL of Buserelin acetate resulted in lower pregnancy and kindling rates compared to 1 μg of Buserelin acetate inoculated by im injection. Thus, the success of this method depends on the status of the vaginal mucosa and on the extender composition because both influence the absorption of GnRH. Vaginal absorption is affected by the degree of solubility in water and molecular weight of the drug (Hussain and Ahsan, 2005) and Quintela et al. (2004) suggested that a fraction of the GnRH analog can be lost by seminal backflow.

At the same time, it should be considered that the molecules used in intravaginal insemination (both GnRH and excipients) can affect sperm physiology. In a previous study, Dal Bosco et al. (2012) verified that the dose of Lecirelin and the volume of the seminal dose greatly affects the ovulation rate and toxicity of the excipient (benzilic alcohol) toward sperm.

Therefore, the aim of the present study is to evaluate the following: (1) the pituitary and ovarian responses of rabbit and (2) the effect on the main sperm traits of Lecirelin diluted with different excipients.

2. Materials and methods

2.1. Animals, housing and diet

A total of 100 multiparous New Zealand White female rabbits were used in this trial. The rabbits were housed in individual cages under a constant photoperiod of 16 h light per day, with a temperature ranging from 15 to 28 °C and a relative humidity of 60–75%. The rabbits were fed *ad libitum* with a commercial diet containing 18.7% crude protein, 14.7% crude fiber, 4.8% fat and 10.9 kcal/kg digestible energy. Water was also available *ad libitum*.

All of the experimental procedures used in this study were approved by the Animal Ethics Committee of the Polytechnic University of Madrid and were in compliance with the Spanish guidelines for the care and use of animals in research (BOE, 2013).

2.2. Experimental design

2.2.1. Experiment 1

All of the rabbits were synchronized 48 h before AI using a prostaglandin treatment and inseminated using a seminal dose containing 10 ± 1 million spermatozoa in 0.5 mL of diluent (Castellini and Lattaioli, 1999). To induce ovulation, the GnRH super-analog Lecirelin acetate (6-(3-methyl-D-valine)-9-(N-ethyl-L-prolynamide)-10-deglycinamide) (Dalmarelin®, Fatro, Italy) was used. Rabbits were divided into 5 homogeneous groups (20 rabbits per group) and submitted to different treatments:

- *Control group*: 5 μg/rabbit of Lecirelin acetate diluted in benzilic alcohol (20 mg/mL), injected (0.2 mL) intramuscularly at the same time as the insemination.
- *Lecirelin group*: 5 μg/rabbit of pure Lecirelin acetate, without any excipient, administered intravaginally by addition to the seminal dose (0.2 mL).
- *Lecirelin BA group*: 5 μg/rabbit of Lecirelin acetate diluted in benzilic alcohol (20 mg/mL), administered intravaginally by addition to the seminal dose (0.2 mL).
- *Lecirelin BAc group*: 5 μg/rabbit of Lecirelin acetate diluted in benzoic acid (1.7 mg/mL), administered intravaginally by addition to the seminal dose (0.2 mL).
- *Lecirelin PA group*: 5 μg/rabbit of Lecirelin acetate diluted in parabens (sodium methyl p-hydroxybenzoate 1.5 mg/mL and sodium propyl p-hydroxybenzoate 0.15 mg/mL), administered intravaginally by addition to the seminal dose (0.2 mL).

2.2.2. Experiment 2

An *in vitro* study was performed to test the effect of Lecirelin additives on sperm. Six male rabbits (10 months of age) were used for collecting semen 3 consecutive times separated by 15 min intervals. The pooled ejaculates were diluted in modified Tyrode's albumin lactate pyruvate buffer (TALP) at a sperm concentration of 20 million spermatozoa/mL.

The semen samples were divided into 5 equal volumes and subjected to the following treatments: semen diluted only in TALP (Control group) or semen diluted in TALP with 5 μg of pure Lecirelin acetate (Lecirelin group), with benzilic alcohol (Lecirelin BA group), with benzoic acid (Lecirelin BAc group) or with parabens (Lecirelin PA group). All of the samples were incubated at 37 °C in 5% CO_2 for 30 min until the kinetics and chlortetracycline (CTC) analysis were performed.

2.3. Blood sampling and hormonal analysis

Blood samples were collected in non-heparinized tubes immediately prior to (−60, −30 and 0 min) and (30, 60, 90, 120 and 180 min) after insemination. A catheter was fixed in the central ear artery before sampling to minimize the effects of handling stress on the hormone concentrations. After collection, the blood was centrifuged at 1200 × *g* for 10 min at 4 °C and serum was stored at −20 °C.

Plasma LH concentrations were determined by a homologous ELISA method validated for rabbits and described by Rebollar et al. (2012). The lowest concentration of RbLH that could be distinguished from the zero concentration was 0.78 ng/mL. The intra-assay coefficient of variation of the analysis was 5.2%. The inter-assay precision calculated for the nine replicate measurements of the coefficient of variation for pools of high and low concentrations was 3.1 and 6.84, respectively. The accuracy of the EIA, determined by measuring the recovery rates of known amounts of RbLH (5, 25, and 125 ng/mL) added to different plasma samples was 90.0%, 96.0%, and 88.6% for low, medium and high values, respectively. In addition, 10 rabbits from each group were subjected to blood sampling to determine their blood progesterone concentration. Blood samples were collected once a week over 4 weeks into EDTA tubes. Immediately after collection, the blood was centrifuged at $3000 \times g$ for 15 min and the plasma was stored at −20 °C until progesterone analysis by radioimmunoassay according to the protocol reported by Gobbetti et al. (1992).

2.4. Ovarian status

To analyze the ovarian status, 10 rabbits per group were euthanized by an intravenous over-dose of Tanax (Hoechst, Frankfurt, Germany) 7 days after AI. The number of pre-ovulatory follicles, mature follicles, hemorrhagic follicles, corpora lutea and flushed embryos from the uterine horns were counted. The ovulation and fertility rates were calculated as follows:

$$\text{Ovulation rate} = 100 \times (\text{number of females that ovulated}/\text{number of AI})$$

$$\text{Fertility rate} = 100 \times (\text{number of kindling}/\text{number of AI})$$

2.5. Evaluation of sperm kinetic characteristics

Kinetic characteristics were analyzed on a computer-assisted semen analyzer (CASA, model ISAS® 4.0, Valencia, Spain). This system consisted of a negative phase contrast optics system (Olympus CH-2) equipped with a Sony CCD camera. The set-up parameters were previously established (Castellini et al., 2011) and the acquisition rate was set at 100 Hz. For each sample, two drops and six microscopic fields were analyzed for a total of 300 spermatozoa. The recorded sperm motion parameters were motility (percentage of motile spermatozoa, %), and curvilinear velocity (VCL, the sum of the incremental distances moved by the spermatozoa in each frame along the sampled path divided by the time, μm/s).

2.6. CTC fluorescence assay

Chlortetracycline (CTC) was assessed in accordance with the protocol described by Kaul et al. (1997). The CTC solution was made by dissolving 750 μM of CTC-HCl in a buffer containing 20 mM Tris–HC1, 130 mM NaCl and 5 mM cysteine–HCl, pH 7.0. The tubes were wrapped with black foil to prevent exposure to light and stored at 4 °C. Fresh solutions were made for daily use. Sperm suspensions (100 μL) were pipetted into 1.5 mL Eppendorf tubes, to which 100 μL of stock CTC was added. The suspension was mixed thoroughly by gentle vortexing. The cells were then immediately fixed in 8 μL of 12.5% (w/v) paraformaldehyde in 0.5 mM Tris HCl buffer, pH 7.5. The slides were prepared by placing 10 μL of the fixed sperm suspension on a slide with one drop of 0.22 M DABCO dissolved in glycerol: PBS (9:1) to reduce fading of the fluorescence. A coverslip was placed on top of the slide and the sperm cells were gently compressed making it possible to remove any excess fluid. The slides were sealed with colorless nail varnish and examined with an epifluorescence microscope (OLYMPUS-CH2 excitation filter 335–425 nm). A total of 300 spermatozoa per sample were analyzed.

Three different fluorescence patterns were detected: fluorescence over the entire head, which is characteristic of uncapacitated acrosome-intact cells; fluorescence on the anterior portion of the sperm head and a dark band in the post acrosomal region, which is characteristic of capacitated acrosome-intact cells; and an absence of fluorescence on the sperm head, which is characteristic of capacitated acrosome-reacted cells (AR).

2.7. Statistical analysis

Data were assessed using the generalized linear model (GLM) procedure of STATA (StataCorp, 2005). The Duncan multiple comparisons test ($P < 0.05$) was used to compare the effect of Lecirelin excipients both in females and in the male sperm used for insemination. The plasma concentrations of LH and progesterone were analyzed by nonlinear (NL) regression. Fractional polynomial regression (STATA – fracpoly procedure) was performed on different data sets (pooled data or separate for treatments). The probability that the NL curves were different was tested with the following algorithm (Glantz and Slinker, 1990):

$$F = \frac{(\text{SS combined} - \text{SS separate})/(\text{DF combined} - \text{DF separate})}{\text{SS separate}/\text{DF separate}}$$

Using the equation below, the F ratio and the corresponding P value were determined.

Differences in ovulation and fertility rates were evaluated by the X^2 FREQ procedure.

3. Results

3.1. Experiment 1

Fig. 1 shows the regression curve for LH concentrations. The pre-ovulatory peak of LH (222.2 ± 12 ng/mL) for the Control group was detected 30 min after AI, which was earlier than for the other iv treatments ($F = 7.70$, $P = 0.0005$). The intravaginal addition of Lecirelin produced lower LH concentrations over time, with an increase 120 min after AI (145.6 ± 12 ng/mL in Lecirelin group).

The weekly variation of progesterone concentrations is reported in Fig. 2. The progesterone concentration for the Control group increased 7 days after AI, plateaued and

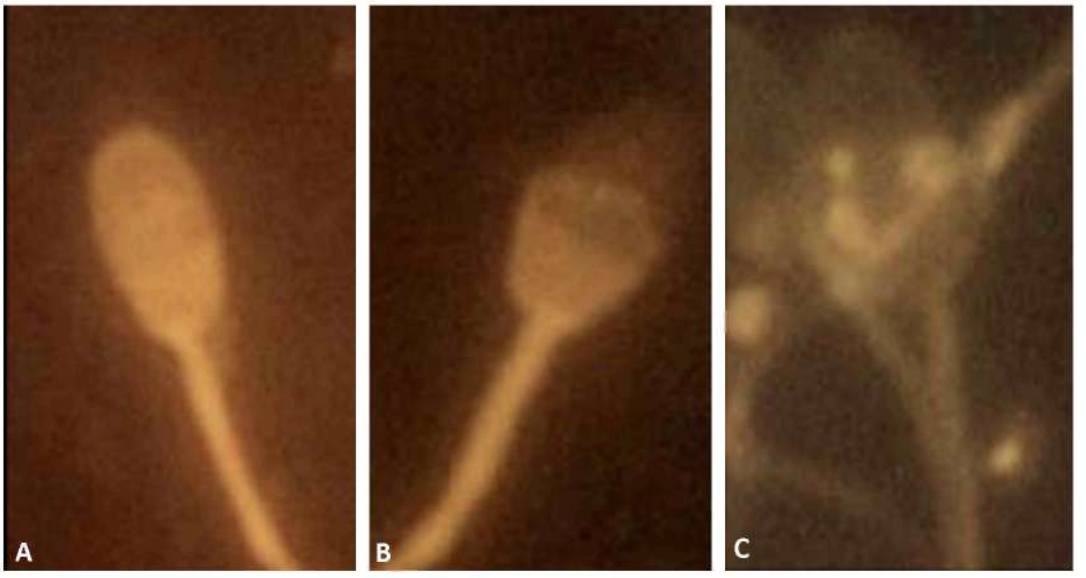

Fig. 1. CTC fluorescence assay. (A) Head characteristic of uncapacitated acrosome-intact cells; (B) anterior portion of the sperm head and a dark band in the post acrosomal region, characteristic of capacitated acrosome-intact cells; and (C) absent fluorescence on the sperm head, which is characteristic of capacitated acrosome-reacted cells (AR).

then decreased after 14 days. The shape of the plasma progesterone curve for the other groups was different ($F = 27.22, P = 6 \times 10^{-10}$), showing an increase 7 and 14 days after AI and decreasing to basal concentrations thereafter (Fig. 3).

No significant differences in the numbers of preovulatory, mature and hemorrhagic follicles were registered between the Control group and the rabbits intravaginally treated with Lecirelin (Table 1). The highest and lowest ovulation rates were obtained in the Control and Lecirelin PA groups, respectively. In the rabbits that ovulated, a comparable number of corpora lutea and embryos were observed in all of the groups.

The highest fertility rates were observed in the Control group, Lecirelin with no excipient and Lecirelin Bac. The lowest fertility rates were observed for Lecirelin PA and Lecirelin BA.

3.2. Experiment 2

Table 2 shows the variation in motility rate, curvilinear velocity and CTC pattern depending on the excipient used. The lowest motility rate and curvilinear velocity were observed in spermatozoa from the Lecirelin BA group. The highest curvilinear velocities were observed in the Control and pure Lecirelin groups. The addition of Lecirelin with or without excipients caused a significant decrease in the percentage of intact cells in the seminal samples, thereby increasing the presence of capacitated spermatozoa. The highest numbers of capacitated and acrosome reacted sperm was observed in seminal samples from the Lecirelin PA Group. The lowest numbers of capacitated and acrosome reacted sperm were observed for the Control and Lecirelin treatments (also in Lecirelin BAc for capacitated spermatozoa).

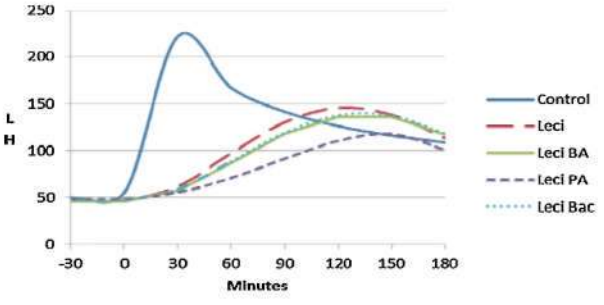

Fig. 2. Regression curve of serum LH concentration (ng/mL) in doses before and after ovulation induction with 5 μg/dose Lecirelin im (Control group) or diluted in the seminal dose with different excipients and inoculated intravaginal (Leci: 5 μg/dose of pure Lecirelin acetate, without any excipient without excipient; Leci BA: 5 μg/dose of Lecirelin acetate diluted in benzilic alcohol; Leci PA: 5 μg/dose of Lecirelin acetate diluted in parabens; Leci Bac: 5 μg/dose of Lecirelin acetate diluted in benzoic acid). $N = 20$ per group.

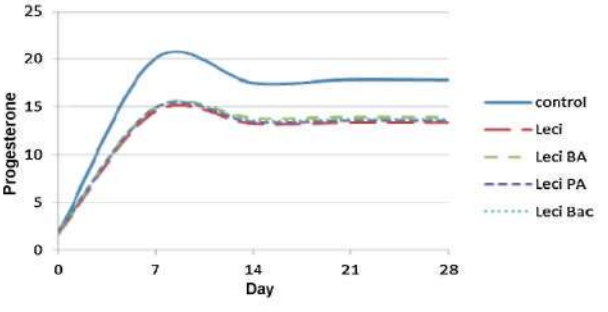

Fig. 3. Regression curve of weekly progesterone concentrations (ng/mL) in pregnant doses after AI and ovulation induction with 5 μg/dose Lecirelin im (Control group) or diluted in the seminal dose with different excipients and inoculated intravaginal (Leci: 5 μg/dose of pure Lecirelin acetate, without any excipient without excipient; Leci BA: 5 μg/dose of Lecirelin acetate diluted in benzilic alcohol; Leci PA: 5 μg/dose of Lecirelin acetate diluted in parabens; Leci Bac: 5 μg/dose of Lecirelin acetate diluted in benzoic acid). $N = 20$ per group.

Table 1
Ovarian status of doses after one week from AI and ovulation induction with 5 μg/dose Lecirelin im (Control group) or diluted in the seminal dose with different excipients.

	Preovulatory follicles (*n*)	Mature follicles (*n*)	Hemorrhagic follicles (*n*)	Ovulation rate % (*n*)	Corpora lutea[x] (*n*)	Embryos[y] (*n*)	Fertility rate % (*n*)
Control	7.2	15.8	0.2	100 (10)[c]	9.4	9.0	80 (8)[b]
Lecirelin no excipient	9.0	17.0	0.3	90 (9)[bc]	9.0	9.0	80 (8)[b]
Lecirelin + benzilic alcohol	10.5	20.4	0.5	70 (7)[ab]	8.5	6.9	40 (4)[a]
Lecirelin + benzoic acid	12.9	19.3	0.0	80 (8)[b]	12.0	8.5	70 (7)[b]
Lecirelin + parabens	10.5	15.8	0.0	60 (6)[a]	8.5	7.0	50 (5)[a]
SED/X^{2*}	1.0	2.4	0.1	10*	1.3	0.8	25*

$N = 10$ per group; [a–c]$P < 0.05$; [x,y] mean of ovulated and pregnant does, respectively.
* X^2.

Table 2
In vitro seminal traits of sperm diluted with 5 μg Lecirelin alone or with different excipients.

	Motility rate (%)	VCL (μm/s)	Intact sperm (%)	Capacitated (%)	Acrosome reacted (%)
Control	85.0[b]	201[c]	77.5[d]	18.0[a]	4.5[a]
Lecirelin no excipient	82.5[b]	210[c]	56.5[c]	40.5[b]	3.0[a]
Lecirelin + benzilic alcohol	19.0[a]	120[a]	36.5[b]	54.2[c]	8.8[b]
Lecirelin + benzoic acid	82.0[b]	177[b]	50.5[c]	40.5[b]	9.0[b]
Lecirelin + parabens	70.5[b]	178[b]	8.0[a]	68.3[d]	23.7[c]
SED	1.0	2.4	10	1.3	0.8

$N = 10$ sample per group; [a–d]$P < 0.05$.

4. Discussion

The present study clearly demonstrates that the intravaginal administration of Lecirelin (5 μg/rabbit) alone or diluted with different excipients, induced a retarded pre-ovulatory peak of LH compared to the control group (120 *vs* 30 min). Similar results have been obtained in relation to the time at which the pre-ovulatory peak is observed. Quintela et al. (2004) reported that rabbits treated with another intravaginal analog (Buserelin) showed an earlier LH peak (60 min post AI) compared to the control group (90 min post AI). Rebollar et al. (2012) showed that the LH peak occurred 60 min after both intramuscular and intravaginal administration of Buserelin in female rabbit. Moreover, the ovulation rate obtained in the present study with im injection of Lecirelin or intravaginal Lecirelin alone was similar to the rate observed by Rebollar et al. (2012).

Thus, our study demonstrates that ovulation can be induced in rabbits by the vaginal absorption of Lecirelin included in the seminal dose. The delay to induce the pituitary response in the intravaginal groups is likely explained by the ability of the vagina to absorb the excipients.

Quintela et al. (2004) suggested that an important fraction of the hormone delivered in the inseminated dose could be lost or not absorbed due to seminal backflow. Moreover, seminal plasma of several species has been reported to have aminopeptidase activity due to proteolytic enzymes. These factors could alter the effectiveness of GnRH analogs absorbed *via* the vaginal mucosa (Vicente et al., 2011).

The low fertility rates in the Lecirelin PA and Lecirelin BA groups were mainly related to Lecirelin dilution in the excipients.

This finding is confirmed by the *in vitro* sperm experiment. Consequently, this experiment clearly demonstrated that the addition of excipients also affects sperm traits and this potentially fertility rate. Excipients are added to protect, stabilize or increase the availability of active compounds; however, the effect of such excipients on vaginal absorption and sperm traits should also be considered (Lee et al., 1997).

Other studies have shown the negative effects of parabens on human sperm vitality (Song et al., 1989) and suggested their use as vaginal contraceptive agents. To our knowledge there is no information on the effect of GnRH excipients on sperm capacitation and AR.

5. Conclusion

In conclusion, the results of this study show that intravaginal administration of Lecirelin can simplify rabbit insemination procedures. However, the use of excipients such as benzilic alcohol, benzoic acid and parabens reduces the fertility rate. Therefore, additional studies are needed to determine the optimal GnRH dose and excipient concentrations that provide an economically efficient and physiologically effective ovulation induction procedures in rabbits.

Conflict of interest

None declared.

Acknowledgements

This study was supported by FATRO S.p.A. - Industria Farmaceutica Veterinaria 40064 Ozzano Emilia (BO) - Via Emilia, 285 and CICYT (AGL-2011-23822).

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